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# Isolation, Screening, and Characterization of Methane-Utilizing Bacteria From the Sediment of Lowland Rice

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**Abstract.**
Methane is a major greenhouse gas that contributes to climate change. Methanogen and methanotrophic group microbes play a role in methane emissions in lowland rice fields. Methane is produced by methanogenic bacteria that decompose organic matter in anaerobic conditions. These bacteria will be active if the soil is inundated for an extended period of time. Some of this methane will be oxidized by methanotrophic bacteria in the rhizosphere. The researchers aimed to isolate, screen, and characterize methane-utilizing bacteria in lowland rice sediment from several Indonesian provinces. 27 methane- utilizing bacteria were isolated from rice field sediments in Lampung, West Java, and East Nusa Tenggara Province. Six of them had the potential to reduce methane emissions by more than half. A *pmoA*-like gene could be found in all of the selected isolates. The bacterial isolates were identified as *Mycobacterium senegalense*, *Bacillus marisflavi*, *Bacillus methylotrophicus*, *Flavobacterium tirrenicum*, *Providencia stuartii*, and *Rhizobium rhizoryzae* after characterization and identification with the Biolog OmniLog® ID system. These were all capable of nitrogen fixation, phosphorus solubilization, and IAA production. These isolates have the potential to be used as biofertilizers and methane mitigation agents.

**Keywords:** biofertilizers, greenhouse gas, lowland rice, methane-utilizing bacteria

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## 1. Introduction

Methane is known as a greenhouse gas that contributes to methane emissions. Most methane gas in the atmosphere comes from microbiological processes, including rice fields [1]. Lowland rice cultivation is high enough to contribute to methane emissions, more than 10% of the total global methane [2]. Inundation in rice fields causes the formation of methane through anaerobic decomposition. More than 80% of methane is released from the rice fields into the atmosphere through the aerenchyma and the intercellular spaces of rice plants as a medium for methane transport.

Methane is produced entirely by methanogenic bacteria, which decompose organic matter anaerobically [3]. These bacteria will be active if the soil is inundated in the long term. Some of the methane will be oxidized by methanotrophic bacteria in the rhizosphere. Research on bacteria that can reduce methane emissions always refers to methane-oxidizing bacteria or methanotrophs. But, Jhala *et al*. (2014) [4] isolated non-methanotrophic bacteria from several rice field ecosystems in India to reduce methane emissions. The bacteria were then classified as methane-utilizing bacteria, some of them have the *pmoA* gene encoding the cluster subunit of methane monooxygenase.

The use of biofertilizers containing methane emission-reducing bacteria is one of the mitigation efforts. These bacteria could promote rice growth and yield and compete with methane-producing bacteria for substrates [5]. This study aims to isolate, screen, characterize and identify methane-utilizing bacteria from several rice rhizospheres in three provinces in Indonesia, which was able to reduce methane emissions and has properties as a biofertilizer.

## 2. Methodology

### 2.1. Samples Collection

Sediments samples were taken in several Indonesian lowland rice ecosystems in three provinces, i.e., West Java, Lampung, and East Nusa Tenggara.

### 2.2. Isolation of Methane-Utilizing Bacteria

Methane-utilizing bacteria were isolated on NMS (Nitrate Mineral Salts) solid media enriched with 1% (v/v) methanol as suggested by Wilkinson (1970) [6]) then incubated at room temperature for 7-14 days. The single colonies of isolates were then screened and biochemically characterized.

### 2.3. Ability to Reduce Methane Emissions

Test the potential reduction of methane emissions from isolates was made by measuring the concentration of methane remaining in the culture headspace using a gas chromatography device. A total of 10% (v/v) of bacterial inoculum was inoculated into 6.3 ml of liquid NMS medium in a 14 ml tube covered by a butyl rubber septum. The gas composition in the headspace tube is made up of 50% methane and 50% air as a

control used a tube containing 7 ml of the same media. Incubation was conducted for 7-14 days on at room temperature and in dark conditions. Methane gas measurements in the headspace were carried out using a gas chromatography technique described by Kumaraswamy *et al.* (2001) [7]. Incubation was carried for 14 days on the shaker at room temperature (27-30$^{o}$C). Methane concentration was measured every four days during the incubation period. Gas samples were taken in the headspace when incubation was complete. The amount of reduction in the methane concentration in the headspace at the measurement interval was used to calculate the methane oxidation activity. Selected isolates that have high ability to reduce methane emissions were then characterized as plant growth-promoting rhizobacteria (PGPR).

## 2.4. Detection of Bacterial Methanotroph Metabolism Gene

One of the key enzymes in the methane metabolic pathway, the *pmoA*-like gene, was used to detect isolates by PCR [8]. Bacterial genomic DNA was isolated using the alkaline lysis method [9]. PCR was performed using specific primers A189F (GGN-GACTGGGACTTCTGG) and mb661 (CCGGMGCAACGTCYTTACC). The PCR reaction was carried out for 30 cycles with the following conditions: initial step at 94$^{o}$C for 3 minutes, denaturation at 94$^{o}$C for 90 seconds, annealing 56$^{o}$C for 45 seconds, elongation 72$^{o}$C for 1 minute, and final extension period of 2 minutes at 72$^{o}$C.

## 2.5. In vitro Screening of Bacterial Isolates for their Plant Growth Promoting Traits

### 2.5.1. Nitrogenase Activity

The activity of the nitrogenase enzyme was measured using the acetylene reduction test [10] . Bacteria were grown in 10 ml test tubes containing 5 ml of semisolid medium without N for 2 days at room temperature. At the end of incubation, each test tube was tightly closed with a rubber stopper. A total of 1 ml of air in the tube was replaced with 1 ml of acetylene gas, then incubated at room temperature for 1 hour. Ethylene production was determined using gas chromatography equipped with a hydrogen flame ionization detector and a Porapack N column.

### 2.5.2. Phosphate Solubilizing Activity

Bacteria were inoculated on Pikovskaya solid medium [11] by droplet technique, then were incubated at room temperature for 3-7 days. The clear zone around the colony indicates the ability of the bacteria to solubilize phosphate.

### 2.5.3. Indole Acetic Acid (IAA) Production

Production of IAA was determined using the Salkowski method modified by Glickmann and Dessau (1995) [12]. Bacteria were grown in NMS liquid medium enriched with 5 mM tryptophan, then incubated at room temperature for 7 days. Two-milliliter supernatant was added with 2 mL of Salkowski's reagent, then incubated for 1 hour at room temperature under dark conditions. The pink color formed indicates the production of IAA. The absorbance was measured using a UV-VIS spectrophotometer at a wavelength of 530 nm.

### 2.5.4. Biochemical Characterization by Biolog OmniLog® ID System

This characterization aims to determine the species or genus of bacterial isolates. Bacterial culture was grown on the Biolog Universal Growth plate. 150 µl microbial suspensions were pipetted into each microplate well, then incubated at 37$^{o}$C for 24 hours. Positive or negative reactions were characterized by changes in color in the well. A positive reaction was marked by changing the color of the well to purple, while a negative reaction is marked by not changing the well's color. The microplate was read by the Biolog MicroStation TM system and then compared with microbial data in the database to determine microbial species identification [13].

## 3. Result and Discussion

We obtained 27 bacterial isolates from 3 provinces (Table 1). The isolates were grown on NMS medium + 1% methanol. All isolates could reduce methane emissions by more than 20%, six of them could even reduce methane gas emissions by more than 50%. Six potent isolates that have the highest ability to reduce methane emissions vis. LM1 (-60.16%), LM17 (-51.26%), LM18 (-60.68%), LM22 (-45.16%), BMU (-57.60%), and N2P4 (-88.10%) were chosen for further study.

Table 1: Methane-Utilizing Bacteria Isolated from Lowland Rice Fields

| Origin of Sample | Total Isolates | Isolates Code |
|---|---|---|
| East Lampung District, Lampung | 12 | LM1, LM2, LM 4, LM5, LM7, LM8, LM12, LM13, LM17, LM18, LM22, LM25 |
| Bogor District, West Java | 4 | BMM, BMU, BMP, BMK |
| Indramayu District, West Java | 6 | I1.12, I1.14, I1.19, I1.29, I2.14, I2.19 |
| Belu District, East Nusa Tenggara | 5 | N2P4, N3P1a, N3P1b, N4P4, N11P4 |

The commonly tested biofertilizer traits are the ability to solubilze phosphate, the production of phytohormones indole acetic acid (IAA), and can fix nitrogen. The ability of rhizobacteria to solubilize insoluble phosphate can increase the availability of phosphorus for plants to increase plant growth and yield [14], while IAA is the essential phytohormone that could promote root development and uptake of nutrients [15]. Nitrogen fixation ability is an essential criterium for the selection of potential PGPR. Screening on the ability to replicate on media without N, forming a clear zone on Pikovskaya media, and producing IAA showed varying results between isolates (Table 2). Not all isolates could solubilize P, but all isolates could grow on N-free media and produced IAA. These isolates' nitrogenase activity varying between 41.798 to 61.921 nmol $C_2H_2$ $ml^{-1}$ $h^{-1}$.

Table 2: *In vitro* Screening of Bacterial Isolates for their Plant Growth Promoting Traits.

| Strains | Nitrogenase Activity (nmol $C_2H_2ml^{-1}$ $h^{-1}$) | Solubilize P | IAA (µg $mL^{--1}$) |
|---|---|---|---|
| LM1 | 91.8 | + | 52.192 |
| LM17 | 53.4 | + | 51.847 |
| LM18 | 74.3 | + | 61.921 |
| LM22 | 92.9 | + | 41.798 |
| BMU | 68.1 | + | 52.094 |
| N2P4 | 76.0 | + | 61.995 |

## 3.1. Detection of Methane Metabolism Gene

Amplification of *pmoA* gene using primer A189/mb661 yielding PCR products of approximately 473-bp size (Figure 2).

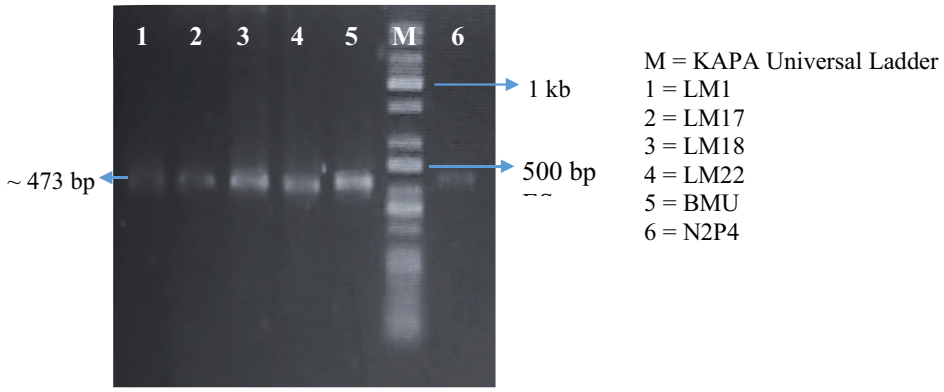



**Figure** 1: The *pmoA* gene amplification profile of methane-utilizing bacteria strains.

## 3.2. Morphological Characteristic of Isolates

The characteristics of each bacterial colony are summarized in Table 3. Isolates LM1, LM17, LM22, and N2P4 were Gram positive, whereas isolate LM18 and BMU were found to be Gram negative.

Table 3: Colonial and morphological characterization of strains on NMS media with 1% methanol.

| Characteristics | Strains | | | | | |
|---|---|---|---|---|---|---|
| | **LM1** | **LM17** | **LM18** | **LM22** | **BMU** | **N2P4** |
| Shape | Circular | Irregular | Irregular | Circular | Irregular | Circular |
| Edge | Smooth | Wavy | Wavy | Smooth | Wavy | Smooth |
| Consistency | Mucoid | Mucoid | Mucoid | Mucoid | Mucoid | Mucoid |
| Elevation | Flat | Flat | Flat | Flat | Flat | Flat |
| Opacity | Opaque | Opaque | Opaque | Opaque | Opaque | Translucent |
| Pigmentation | White | Pink | Pink | Pink | Light violet | White |
| Gram staining | Positive | Positive | Negative | Positive | Negative | Positive |

## 3.3. Biochemical Characterization and Identification

The metabolic profiles of isolates LM1, LM17, LM18, LM22, LMU, and N2P4 tested on BIOLOG GP2 Microplates are compiled in Table 4.

All bacterial isolates showed different similarity (SIM) and distance (DIS) values after incubation for 24 hours (Table 5).

Table 4: Similarity and Distant of Methane-Utilizing Bacteria.

| | **LM1** | **LM17** | **LM18** | **LM22** | **BMU** | **N2P4** |
|---|---|---|---|---|---|---|
| Similarity | 0.604 | 0.757 | 0.749 | 0.749 | 0.697 | 0.662 |
| Distant | 4.581 | 4.654 | 4.767 | 4.800 | 5.044 | 4.361 |

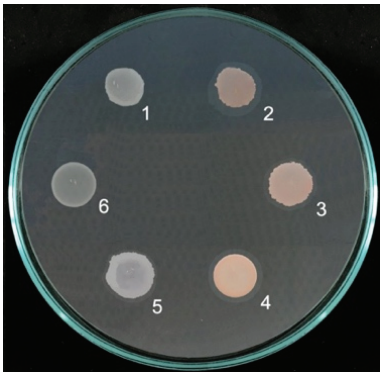
| Well | Carbon source | Strain | | | | | |
|---|---|---|---|---|---|---|---|
| | | LM1 | LM17 | LM18 | LM22 | BMU | N2P4 |
| A1 | Control negative | - | - | - | - | - | - |
| 2 | Dextrine | - | + | + | + | + | + |
| 3 | D-Maltose | + | + | + | + | + | + |
| 4 | D-Trehalose | + | + | + | + | + | + |
| 5 | D-Cellobiose | + | + | + | + | + | + |
| 6 | Gentiobiose | + | + | + | + | + | + |
| 7 | Sucrose | + | + | + | + | + | + |
| 8 | D-Turanose | + | + | + | + | + | + |
| 9 | Stachyose | - | + | + | - | + | + |
| 10 | Control positive | + | + | + | + | + | + |
| 11 | pH 6 | + | + | + | + | + | + |
| 12 | pH 5 | + | + | + | + | - | + |
| B1 | D-Raffinose | - | + | + | - | + | + |
| 2 | α-D-Lactose | - | + | + | - | + | + |
| 3 | D-Melibiose | + | + | + | + | + | + |
| 4 | β-Methyl D-Glucoside | + | + | + | - | + | + |
| 5 | D-Salicin | + | + | + | + | + | + |
| 6 | N-Acetyl D-Glucosamine | + | + | + | + | + | + |
| 7 | N-Acetyl β-D-Mannosamine | - | + | - | - | - | - |
| 8 | N-Acetyl D-Galactosamine | + | + | + | + | + | + |
| 9 | N-Acetyl Neuraminic acid | - | + | + | - | - | - |
| 10 | 1% NaCl | + | + | + | + | + | + |
| 11 | 4% NaCl | + | + | + | + | + | + |
| 12 | 8% NaCl | - | - | - | - | - | + |
| C1 | α- D-Glucose | + | + | + | + | + | + |
| 2 | D-Mannose | + | + | + | + | + | - |
| 3 | D-Fructose | - | + | + | + | + | + |
| 4 | D-Galactose | + | + | + | - | + | + |
| 5 | 3-Methyl Glucose | - | + | + | - | - | + |
| 6 | D-Fucose | - | + | + | + | + | - |
| 7 | L-Fucose | + | + | + | + | + | + |
| 8 | L-Rhamnose | - | + | + | - | + | + |
| 9 | Inosine | - | + | + | + | + | + |
| 10 | 1% Sodium Lactate | + | + | + | + | + | + |
| 11 | Fusidic acid | + | + | + | + | - | + |
| 12 | D-Serine | + | - | + | + | - | + |
| D1 | D-Sorbitol | - | + | + | - | + | + |
| 2 | D-Mannitol | - | + | + | + | + | + |
| 3 | D-Arabitol | + | + | + | - | + | + |
| 4 | Myo-Inositol | + | + | + | - | + | + |
| 5 | Glycerol | - | + | + | + | + | + |
| 6 | D-Glucose-6PO$_4$ | - | + | + | - | + | + |
| 7 | D-Fructose-6PO$_4$ | - | + | + | + | + | + |
| 8 | D-Aspartic Acid | - | + | - | + | - | + |
| 9 | D-Serine | + | - | + | - | - | - |
| 10 | Troleandomycin | + | + | + | + | + | + |
| 11 | Rifamycin SV | + | + | + | + | + | + |
| 12 | Minocycline | - | - | + | + | - | - |

| Well | Carbon source | Strain | | | | | |
|---|---|---|---|---|---|---|---|
| | | LM1 | LM17 | LM18 | LM22 | BMU | N2P4 |
| E1 | Gelatine | + | + | + | + | - | + |
| 2 | Glycyl-L-Proline | + | + | + | + | + | + |
| 3 | L-Alanine | + | + | + | + | + | + |
| 4 | L-Arginine | + | + | + | - | + | + |
| 5 | L-Aspartic Acid | + | + | + | + | + | + |
| 6 | L-Glutamic Acid | + | + | + | + | + | + |
| 7 | L-Histidine | + | + | + | + | + | + |
| 8 | L-Pyroglutamic Acid | + | + | + | + | + | + |
| 9 | L-Serine | + | + | + | + | + | + |
| 10 | Lincomycin | + | + | + | + | + | - |
| 11 | Guanidine HCl | + | + | + | + | + | + |
| 12 | Niaproof 4 | + | - | + | + | + | - |
| F1 | Pectin | + | + | + | + | + | + |
| 2 | D-Galacturonic Acid | + | + | + | + | + | + |
| 3 | L-Galactonic Acid Lactose | - | + | + | - | + | + |
| 4 | D-Gluconic Acid | + | + | + | + | + | + |
| 5 | D-Glucuronic Acid | - | + | + | + | + | + |
| 6 | Glucuronamide | - | + | + | + | - | - |
| 7 | Mucic Acid | - | + | + | + | - | + |
| 8 | Quinic Acid | + | + | + | - | + | + |
| 9 | D-Saccharic Acid | + | + | + | + | - | + |
| 10 | Vancomycin | + | + | + | + | + | + |
| 11 | Tetrazolium Violet | + | + | + | + | + | - |
| 12 | Tetrazolium Blue | + | + | + | + | + | + |
| G1 | ρ-Hydroxy-Phenylacetic Acid | - | + | + | + | + | - |
| 2 | Methyl Pyruvate | + | + | + | + | + | - |
| 3 | D-Lactic Acid Methyl Ester | - | + | - | - | - | + |
| 4 | D-Lactic Acid | + | + | + | + | + | + |
| 5 | Citric Acid | + | + | + | + | - | + |
| 6 | α-Keto-Glutaric Acid | + | + | + | + | + | + |
| 7 | D-Malic Acid | + | + | + | + | + | - |
| 8 | L-Malic Acid | + | + | + | + | + | + |
| 9 | Bromo-Succinic Acid | + | + | + | + | - | + |
| 10 | Nalidixid Acid | + | + | + | + | + | + |
| 11 | Lithium Chloride | + | + | + | + | + | + |
| 12 | Potassium Tellurite | + | + | + | + | + | + |
| H1 | Tween 40 | + | + | + | + | - | + |
| 2 | γ-Amino-Butyric Acid | - | + | + | + | + | + |
| 3 | α-Hydroxy-Butyric Acid | + | + | + | + | + | - |
| 4 | β-Hyroxy-D,L-Butiryc Acid | + | + | + | + | + | + |
| 5 | α-Keto Butyric Acid | - | + | + | + | +/- | - |
| 6 | Acetoacetic Acid | + | + | - | + | + | + |
| 7 | Propionic Acid | + | + | + | + | + | - |
| 8 | Acetic Acid | + | + | + | + | + | + |
| 9 | Formic Acid | + | + | + | + | - | - |
| 10 | Aztreonam | + | + | + | + | + | + |
| 11 | Sodium Butyrate | + | - | - | + | + | + |
| 12 | Sodium Bromate | - | - | - | - | - | - |

**Figure** 2: Biochemical Characterstics of Isolates using BIOLOG OmniLog® ID System.

According to the metabolic fingerprint and microbial database on the OmniLog® Biology ID System, isolate LM1 was identified as *Mycobacterium senegalense*, isolate LM17 could be assigned to *Bacillus marisflavi*, isolate LM18 was proposed as *Providencia stuartii*, and isolate LM22 was identified as *Flavobacterium tirrenicum*. The BMU isolate was suspected as *Rhizobium rhizoryzae*, while the N2P4 isolate could be proposed as *Bacillus methylotrophicus*. They all have different colonial morphologies (Figure 3).

The BIOLOG System was able to identify isolates LM1, LM17, LM18, LM22, BMU, and N2P4 to the species level correctly. Research conducted by Klingler *et al*. (2000) [16] succeeded in identifying 39 of 41 (98%) bacterial cultures up to genus level according to the ATCC reference. Although the most accurate determination of bacterial species identification is based on the results of 16sRNA amplification sequencing, some authors recommend that the BIOLOG System and partial sequencing of the 16S rDNA gene can be used simultaneously to complete an accurate taxonomy of several strains [17].




1 = *Mycobacterium senegalense* LM1
2 = *Bacillus marisflavi* LM17
3 = *Providencia stuarti* LM18
4 = *Flavobacterium tirrenicum* LM22
5 = *Rhizobium rhizoryzae* BMU
6 = *Bacillus methylotrophicus* N2P4

**Figure** 3: Colonial morphological of methane-utilizing bacteria strains on NMS media + 1% methanol (14 days after incubation).

These bacteria were not methanotrophs, but they have been detected to have the *pmoA* gene and reduced methane emissions. This finding has never been reported in Indonesia. Research conducted by Jhala *et al.* (2014)[4] got similar results. Thus, they classified these bacteria as methane-utilizing bacteria. Several studies have recorded that *M. senegalense*, *B. marisflavi*, *Providencia stuartii, F. tirrenicum*, and *R. rhizoryzae* have the ability as biofertilizers for paddy, sorghum, and crucifers [18] [19] [20] [21]. The use of PGPR as a biofertilizer is highly recommended to increase crop yields, quality, and soil fertility.

## 4. Conclusions

The methane-utilizing bacteria were *Mycobacterium senegalense, Bacillus marisflavi, Bacillus methylotrophicus, Flavobacterium tirrenicum, Providencia stuartii, Rhizobium rhizoryzae*. They all could fix nitrogen, solubilize P, and produce IAA. These isolates have the potential as biofertilizers and mitigation agents to reduce methane emissions.

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